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## TABLE OF CONTENTS

Front Cover	page 1
SF 298	2
Foreword	3
Table of Contents	4
Introduction	5
Objectives	7
Body	8
<i>Results:</i> Strategy for expression and screening of ligand induced FLP recombination	8
Fusion point of the estrogen LBD differentially affects hormone/ antihormone action	9
A FLP-E/F estrogen receptor fusion protein binds, but is not activated by antagonists	10
Differences between agonist and antagonist activation are partly dependent on the F domain	11
Repositioning of helix 12 by mutation brings F domain interference with agonist binding	11
Genetically selected mutants relied on selection pressures imposed by the positions of helix 12 and F domain	12
A mutation of Helix 12 blocks activation by Raloxifene	13
Library generation by codon substitution mutagenesis (CSM)	14
Screening assay for altered specificity of ligand binding	14
<i>Discussion:</i> Fusing the estrogen LBD closer to FLP selectively blocks activation by antihormones	16
Different positioning of the LBD helix 12 and the F domain of ER accounts for functional differences between agonists and antagonists	16
Helix 10 causes the inversion phenotype by misplacing helix 12 and the F domain.	17
Inversion mutations of other nuclear receptors	18
The F domain length is conserved	19
Hsp90 complex regulates LBD-fusion proteins	19
<i>Materials and Methods:</i>	20
Conclusions	22
References	24
Figure legends	28
Tables I- IV	32
Figures 1- 12	38

## **A Genetic Screen for Ligand Binding by the Human Estrogen Receptor.**

### **Introduction**

Estrogens regulate target cell proliferation and gene transcription through a pathway of molecular events initiated by binding to the estrogen receptor (ER), a member of the steroid hormone receptor class of nuclear receptors (3,14). A diverse group of antagonistic ligands have been identified which interfere with ER activation (11,23,40). Prominent among these are the clinically relevant antagonists, 4-hydroxytamoxifen (Z-OHT), raloxifene and ICI 182,780. Biochemical and functional work on the mechanisms of antagonist action has led to the recognition that they induce different conformations of the ER ligand binding domain (domain E) than agonists (1) and show a spectrum of activities, presumably a reflection of the multiple steps involved in the ER activation pathway and the diversity of responses elicited (see 23,25). However the molecular basis of the differences between agonist and antagonist activities remains to be elucidated.

Steroid receptors are modular proteins possessing two highly conserved domains, termed the C and E domains (3,14). They are joined by an unstructured and variable D domain sequence (14). Domain C is 66 amino acids long with two zinc fingers, which mediate sequence-specific DNA binding. Domain E, also known as the ligand binding domain (LBD), is about 240 amino acids long (44) and mediates numerous overlapping functions: ligand binding, dimerization, Hsp90 binding, transrepression, transcriptional activation and cellular localization (3,22). Mutations within LBDs have revealed some insight into the structure/function relationship, and the x-ray crystal structures of the related retinoid and thyroid nuclear receptor LBDs present a framework for understanding how LBDs function (6,31,34,39,43). The E domain can be fused onto certain other proteins to impose ligand dependency on protein activity (32). Here we make use of our observation that steroid regulation can be imposed on the enzyme activity of a site specific recombinase, FLP, by expressing FLP/steroid receptor fusion proteins (FLP-LBDs, 30). FLP-LBDs are inactive as recombinases in the absence of a cognate ligand and respond to both agonists and antagonists in a titratable manner (30), resulting in a fixed change in reporter gene DNA which converts ligand binding to an enzyme activity. Thus FLP-LBDs faithfully reflect the initially repressed, unliganded, state of steroid

receptors but do not discriminate between agonists and antagonists. Hence the differences between these two classes of ligands must occur after ligand-induced release from the initially repressed state (30). For example, two domains of the estrogen receptor protein are capable of transcriptional activation functions (AFs): a hormone-independent AF-1 in the A/B domain and the hormone-dependent AF-2 at helix 12 within the E (LBD) domain (20). AF-1 and AF-2 functions vary in importance with individual promoters and cell-types and the primary determinant of AF-2 function depends on whether the bound ligand is an agonist or an antagonist (4,23,24,41). Consequently, FLP-LBDs present assays where ligand activities are measured directly, by site specific recombination, and the multiple steps and specificities involved in ligand-mediated transcriptional responses are circumvented. Here, a FLP-estrogen binding domain fusion protein assay in yeast, where no transcriptional cofactor has been found which interacts with the steroid hormone receptor AF-2 function (4), is used to address conformational differences induced by agonists and antagonists.

The amount of estrogen receptor D domain included in the fusion protein between the FLP and EBD domains influences the activity of the recombinase. If the D domain is omitted, antihormones but not hormones, are unable to activate the fusion recombinase. The ability to distinguish between hormones and antihormones in yeast is extremely important in understanding how ligands activate the estrogen receptor. The objective of this effort is to exploit yeast-based genetic screens of mutated EBD clone banks to find mutations which specifically alter ligand binding, and hence recombination. By use of different ligands and various concentrations, we find specific amino acid changes which alter ligand induced function, thereby defining components of binding and aiding rational ligand design. Combining the EBD fusion point dependence for antihormone action with numerous random mutations of the estrogen binding domain has produced EBDs which show altered or reversed activation by hormones and antihormones.

**Objectives**

This research involves defining the interactions between estrogen ligands and the estrogen binding domain to ascertain a better understanding of ligand binding and function, especially as that relates to hormone and antihormone function. The fusion point dependence for antihormone action in our FLP recombinase/ estrogen binding domain (FLP-EBD) system allows a mechanism to study therapeutically important antihormones, such as tamoxifen and raloxifene, and why/how they act as antihormones. We find that agonists and antagonists position the C-terminal part of the ligand binding domain (helix 12) and the F domain differently, leading to their known opposite effects on ER in transcription. Helix 12 is the most important region for AF-2 function and cofactor binding for transcription. Numerous mutations in the EBD also alter a subset of ligand interactions. This strategy separates multiple functions present in the estrogen binding domain and should further the potential for rational drug design.

## **BODY**

This report covers the grants, DAMD17-94-J-4103 (Sept 96-Aug 97); DAMD17-94-J-4249 (Sept 96-Jan 97; expired). The work to date has involved characterizing FLP recombinase-estrogen receptor hormone binding domain (FLP-EBD) fusion proteins in yeast and screening mutated ligand binding domains for altered ligand interaction. Having developed an improved strategy, I have applied it to (a) continued mutagenic library screening such as presented below, and (b) ligand changes in yeast colony color as a simple way to classify estrogen hormones and antihormones. Using a combination of mutations, I have found that the ligand induced positions of the ligand binding domain helix 12 and the F domain differentiate hormones and antihormones. This surely influences coactivator and corepressor interactions with ER in transcription. I also include work with the medically important antihormones, tamoxifen and raloxifene and find one mutation in helix 12 which affects raloxifene specifically.

### **Results:**

#### **Strategy for expression and screening of ligand induced FLP recombination.**

To regulate the FLP recombinase in yeast, the human ER hormone binding domain (domains D, E, and F; aa 251-595) was fused to the C-terminus of the entire coding sequence (423 aa) of FLP recombinase. The fusion gene was cloned under the control of the GAL10 galactose promoter (Figure 1A). Thus transcription and expression is limited to galactose media, with virtually no expression in glucose media. The fusion gene was inserted into a derivative of pRS315 (38), a single-copy CEN plasmid with the LEU2 selectable marker. Various restriction sites have been introduced into the estrogen LBD coding sequence (aa 306-595 of the human ER) without changing the amino acid sequence to simplify mutated library cloning. Details were presented in previous reports.

To report FLP recombinase activity, a single-copy deletion recombination substrate was integrated at the TRP1 locus. The recombination target includes the constitutive alcohol dehydrogenase (ADH1) promoter directing transcription of the URA3 gene, followed by a polyA signal to terminate RNA Pol II transcription (Figure 1B). The URA3 gene and a SUP11 ochre suppressor tRNA gene are flanked by FLP



recombination targets (FRTs). The URA3<sup>+</sup> gene can be positively or negatively selected for growth. The SUP11 ochre suppressor tRNA gene between the FRTs allows a visual screen for recombination, using the red/white Ade2<sup>+</sup> colony color assay (29), as the tRNA suppresses the ade2-1 ochre allele and gives white colonies. A red pigment accumulates in ade2- cells. Thus recombination is detected by colony color assays or by Southern analysis.

Time course experiments confirmed the galactose control of expression was operating as designed, giving linear recombination between 4 and 10 hours, dependent on galactose for transcription and hormone to derepress the FLP-EBD protein. Hormone concentration experiments with a variety of known estrogen hormones and antihormones confirmed that the response of FLP-EBDs to ligands is a simple reflection of ligand binding by the EBD (30; last year's report). All ligands tested (Fig. 1C), whether hormones or antihormones, induce the FLP-EBD fusion proteins.

### **Fusion point of the estrogen LBD differentially affects hormone/ antihormone action.**

The D domain is thought to be a flexible, unstructured hinge region between the conserved C (DNA binding) and E (ligand binding) domains (6,12,19,34,37,43). We observed that constructs without a D domain were not activated by antihormones, even at very high concentrations (30; last year report).

An Ade2<sup>+</sup> color plate assay for ligand induced recombination was developed as a way to visualize the presence and concentration of ligands (last year report). We tested the FLP-EBD with and without the D domain in the plate assay and confirm that antagonists (Z-OHT, Ral, Tam) as a class, do not activate FLP-EBD without the D domain spacer (Fig. 2, compare WT251 vs. WT304, where 53 aa of D domain have been deleted).

It was possible that the shorter form of the fusion proteins, FLP-EBD304, failed to respond to antihormones due to a specific loss of binding affinity. To address this possibility, we performed *in vitro* ligand binding experiments to measure estradiol and 4-hydroxytamoxifen binding by the FLP-EBD251 and FLP-EBD304 fusion proteins expressed in yeast (Fig. 3). Binding experiments utilized a fixed concentration of radiolabelled <sup>3</sup>H-estradiol (1nM), which was pre-mixed with zero or increasing amounts of unlabelled estradiol (E2; 1nM to 1000nM) or 4-hydroxytamoxifen (Z-OHT; 10nM to 10,000nM). We found that the FLP-

EBD304 form had the same binding affinity (half maximal inhibition, IC<sub>50</sub>) for 4-hydroxytamoxifen (Z-OHT) and estradiol (E2) as the FLP-EBD251 form (Fig. 3). Therefore if the antihormone concentration is sufficient to bind and induce recombination by the FLP-EBD251 form, it should also activate the FLP-EBD304 form, yet no induced recombination was seen, even with 10-100x more antihormone added (Fig. 2; 30). We conclude that the *antihormone* bound FLP-EBD304 protein *in vivo* is not capable of recombination due to steric hindrance with FLP, though the hormone bound FLP-EBD304 is properly folded and active.

To verify that the fusion proteins were not responding differently for trivial, protein stability reasons, Western assays were performed. Extracts of yeast expressing the various FLP-EBDs were prepared and compared with an antibody to the C-terminus of ER (F domain). FLP recombinase/ ER fusion proteins migrate at the expected sizes, and two representative mutant forms of FLP-EBD do not change the amount or the size of protein recovered, implying similar expression and protein stability (further samples, not shown).

#### **A FLP-E/F estrogen receptor fusion protein binds, but is not activated by, antagonists**

As shown before, a FLP-ER fusion protein that included the ER D, E and F domains (FLP-D/E/F) responded to all ligands tested, whether agonist or antagonist (Fig. 2; 30). This demonstrates that both ligand classes release ER from its initially repressed state and that the known differences between their activities are caused by differing specificities later in the pathway of transcriptional activation.

In contrast, the FLP-E/F protein, which has no D domain, was activated by agonists but not by antagonists (Fig 2; 30; and data not shown). This was unexpected since all studies with nuclear receptors show that the E domain is a modular entity that entirely encompasses the function of ligand binding (44). To determine molecular reasons for the differential activity of these two classes of ligands, we constructed a number of mutation variants involving the D, E, and F domains, outlined in Figure 4 and discussed below. Consistent with previous studies, binding experiments using yeast extracts containing FLP-E/F and FLP-D/E/F proteins showed that both fusion proteins bound all agonists and antagonists with affinities close to those of the native estrogen receptor (Fig. 1C; 30). The half maximal inhibition values for various ligands versus labelled estradiol, are diagrammed next to the

corresponding EBD form (Fig. 4). Lack of activation of FLP-E/F by antagonists was not due to a lack of binding.

### **Differences between agonist and antagonist activation are partly dependent on the F domain**

Insight into the molecular basis underlying this difference between binding and activation was found from experiments which examined the role of the estrogen receptor F domain. In contrast to the FLP-E/F fusion protein which was not activated at all by antagonists, the FLP-E fusion protein, derived from FLP-E/F by deletion of the F domain, was partially activated by Z-OHT (Fig. 5A: compare FLP-E/F and FLP-E). Similarly, with FLP-D/E, the absence of the F domain also improved activation by Z-OHT (Fig. 5B: compare FLP-D/E/F with FLP-D/E). These results demonstrate that the difference between antagonist binding and activation was due in part to interference by the F domain. This interference was increased by removal of the D domain, moving the E and F domains closer to FLP and its tetrameric reaction intermediate (7). In contrast, the proper, agonist bound conformation did not result in F domain interference.

### **Repositioning of helix 12 by mutation brings F domain interference with agonist binding**

The x-ray crystal structures of the unliganded RXR and agonist-bound RAR E domains led to a "mouse trap" model of ligand binding by nuclear receptors, which invokes a large repositioning of the C-terminus of the E domain, helix 12 (6,34). It is important to note that no x-ray structures of ligand binding domains so far include the F domain. F domain interference upon antagonist binding could reflect different positioning of helix 12, and consequently the F domain. If so, then altering the position of helix 12 by mutation should invoke F domain interference upon agonist binding. This proved to be the case. Regardless of the inclusion of the "spacer" D domain or not, mutational disruption of helix 12 by proline (L540P) impaired agonist activation (Fig. 5A: FLP-E(L540P)/F; 5B: FLP-D/E(L540P)/F). This impairment was due in part to F domain interference, since deletion of the F domain from either helix 12 mutant protein restored activation (Fig. 5A: FLP-E(L540P); 5B: FLP-D/E(L540P)).

### **Genetically selected activation mutants relied on selection pressures imposed by the positions of helix 12 and the F domain**

In an independent series of experiments to determine why the FLP-E/F protein was resistant to activation by antagonists, we used the connection between recombination and ligand activation to select mutants that switched from agonist activation/antagonist resistance to antagonist activation/agonist resistance. The selection procedure is outlined in Fig. 6A. FLP-E/F was heavily mutated between amino acids 506 and 527 by codon substitution mutagenesis (8,13; previous reports). This region was chosen because it includes helix 11, which is important in agonist contacts in the RAR and TR crystal structures (34,43), and ER amino acid 521, the site of ligand selective ER mutants (10). FLP-E/F mutations that were resistant to activation by estradiol were selected by culturing the mutant libraries in the presence of estradiol while selecting for expression of the URA3 gene and hence, against recombination. Survivors were then cultured in the presence of an antagonist (Z-OHT or raloxifene) without selection, to permit growth after recombination, and then spread on plates to identify single recombinants by a red colony color. Red colonies identify those mutations between amino acids 506 and 527 that are resistant to activation by estradiol but are activated by the antagonists Z-OHT or raloxifene. Candidate FLP-mutant E/F expression plasmids were isolated from red colonies and retested in the recombination reporter host (Fig. 1B) by a colony color assay. Yeast carrying the candidate expression plasmid and the unrecombined recombination reporter were spread as a lawn on a plate to which ER ligands were applied (Fig. 6B). The diameter of the circle of red yeast, induced by diffusing ligand, directly reflects the relative activation of the recombinase fusion by that ligand. Here the plate assay shows the agonist-only activation phenotype of the parent FLP-E/F protein, and for one of the mutants isolated (FLP-E(508E)/F), it shows the inversion phenotype of resistance to agonists, activation by antagonists.

Unexpectedly, no helix 11 mutations were recovered in this screen although 17 independent mutations were identified (Table I). As a control, the known estradiol-resistant, antagonist-inducible G521R helix 11 mutation (10) was tested directly as a FLP-E(G521R)/F protein and was virtually inactive in this protocol (Fig. 6C). We attribute this to the fact that the G521R mutation is both a loss of binding mutation for

estradiol (more than 10,000 fold) and a loss of binding mutation for Z-OHT (approximately 100 fold, Fig. 6C; data not shown) therefore making it too insensitive to ligands to be identified by this analysis. Testing the G521R mutation in the FLP-D/E/F background showed the expected increase in recombinase activity with less F domain interference. Most of the mutations identified affected helix 10 by introducing charged or polar residues into a very hydrophobic part of the  $\alpha$ -helix (Table I). Southern experiments for two other examples of these inversion mutations, exhibit good activation by antagonists and loss of agonist induction (Fig. 7). None of those tested showed substantial differences in binding of either agonists or antagonists (Fig. 1C). These mutations therefore affected estradiol activation, not binding, and so were conceptually similar to the case of antagonists and FLP-E/F (Fig. 5A). Consequently we tested a representative helix 10 mutation to establish whether the F domain was also responsible for interfering with activation.

FLP-E(L508R)/F was partially activated by Z-OHT and not significantly by estradiol (Fig. 8A) as expected from the mutagenesis and the selection protocol used. Activation by both agonists and antagonists was improved when the F domain was deleted (Fig. 8A, FLP-E(L508R); and data not shown). Disordering helix 12 by mutation had a slight effect on estradiol activation in the presence of the F domain (Fig. 8A: compare FLP-E(L508R)/F with FLP-E(L508R, L540P)/F). However the combination of the helix 12 mutation with the removal of the F domain fully restored estradiol activation (Fig. 8A, FLP-E(L508R,L540P)). Plate assay experiments, such as shown in Fig. 6B, also confirm the results and conclusions drawn from the southern assays (data not shown). Since none of these mutations had any significant effect on agonist or antagonist binding, this demonstrates that the helix 10 mutations identified in the selection protocol imposed interference on estradiol activation by structurally repositioning helix 12 and the F domain.

#### **A mutation of Helix 12 specifically blocks activation by Raloxifene.**

Disturbing helix 12 leads to a key determinant of ligand interpretation. In our studies relating agonists/ antagonists, we noticed mutations which specifically altered the response to raloxifene only. Plate assays using the D domain containing, WT251 form show that all ligands activate, and that deletion of the F domain only very slightly

increases the ligand induced recombination (Fig. 9). Any form that includes disruption of the helix 12 (L540P) also specifically blocks any activation by raloxifene (Fig. 9). The region at helix 12 of the LBD is the critical helix for AF-2 function in transcription (9) and disrupts coactivators which bind to steroid hormone receptors (15). This is a key observation to understand the differences between tamoxifen and raloxifene types of antihormones.

A schematic diagram of important agonist vs. antagonist functions and domains is shown in Fig. 10, and is described further in the discussion.

### **Library generation by codon substitution mutagenesis (CSM).**

CSM, an oligonucleotide based method, is the best method available for saturation mutagenesis of a region (8). The initial mutation libraries focused on a region (aa 506-532) previously implicated by random single mutation data to be important for estrogen ligand specificity (Fig. 11). We have since generated libraries covering many of the predicted regions of importance for ligand interaction or specificity (last year report), as judged by the existing x-ray crystal structures of the retinoid receptors, RAR, RXR, and TR (6,34,43). I have not generated a ninth mutation library *per se*, but instead have made site-specific mutations to test particular questions relating to hormone/antihormone function (as discussed). We have numerous mutations in hand to continue further analysis of ligand interaction (see below, Tables II, III).

### **Screening assay for altered specificity of ligand binding.**

After generating a library of mutagenized FLP-EBD constructs, we perform a screening assay to enrich for plasmids containing mutations that are unreactive with a first ligand, yet still retain binding to a second ligand, resulting in recombination (as highlighted in Fig. 6). These plasmids are retested in the parent strain to confirm differential inducibility, as compared to the wild-type sequence, by a set of ligands. Screening of the libraries is ongoing, and the ligand inversion mutations were discussed above. Another type of mutation leads to a super induced phenotype, where the mutated form is more responsive to ligands than the wild-type sequence (Fig. 12), and in this case leads to a specific raloxifene induction also. Table II summarizes a number of alleles already sequenced with characterized phenotype. Mutations which respond as well as the wild-type sequence have amino acid changes

which are very conservative. Weak wild-type phenotypes are still activatable by agonists only, but with a much reduced recombination activity. Some of these may be ligand affinity mutations. Other mutations (denoted wt+) are activated by a wider spectrum of ligands than the wild-type sequence. Altered specificity mutations show a drop or increase in activation by only one or several ligands. Finally a number of mutations block ligand binding to the receptor and do not derepress recombinase activity. These include non-conservative and multiple substitutions, as well as premature terminations within the LBD. A more complete, yet not fully characterized list of mutations throughout much of the LBD is shown in Table III, where fs = frameshifts and Term = termination codons. Work continues with these mutations and screening of libraries.



## **Discussion:**

### **Fusing the estrogen LBD closer to FLP selectively blocks activation by antihormones.**

Previous work on several steroid receptors has implied that different conformations arise after hormone vs. antihormone binding. These conclusions primarily derive from protease clipping assays of the entire ligand binding domain, some 240 amino acids, and are necessarily imprecise (1). Others have shown differences in total hydrophobicity of the domain. In previous work with FLP-steroid receptor fusion proteins, we observed that all ligands, whether agonists or antagonists, served to release the cognate FLP-fusion protein from its initially repressed condition. This is true both in yeast (30) and mammalian cells (unpublished results). Therefore release from the initially repressed condition is concomitant with ligand binding and any differences in activities between agonists and antagonists must lie downstream. However we also observed that estrogen receptor fusion proteins that omitted the D domain, FLP-E/Fs, were activated by agonists but not by antagonists, in spite of the fact that both ligand classes were bound with near wild type affinities (Fig. 1C; 30).

### **Different positioning of the LBD helix 12 and the F domain of ER accounts for functional differences between agonists and antagonists**

In this report, we describe work to understand the observed difference between agonist/antagonist binding and activation. We found that antagonist activation can be partially restored by removal of the F domain, arguing that antagonist binding positions the F domain so that it interferes with FLP recombination. Repositioning the F domain by mutating helix 12 resulted in F domain interference with agonist binding. Genetically selected mutations that inverted agonist activation/antagonist resistance to agonist resistance/antagonist activation were similarly dependent on the positioning of helix 12 and the F domain. This gives new insight to the mechanism of similar phenotypic mutations for several nuclear receptors described in the literature, which had no previous explanation (see below). Thus three aspects important to the difference between ligand binding and activation were identified - the presence of the F domain, the



positioning of helix 12 and the proximity of domain E to FLP recombinase in the fusion protein.

**Helix 10 causes the inversion phenotype by misplacing helix 12 and the F domain.**

Taken together, the data can only be simply explained by steric differences induced by binding of agonists or antagonists, or the mutations employed. In particular, we show that distancing domains E and F from FLP in the fusion protein by including the D domain in between dilutes interference in all cases. This discounts simple explanations that rely on intermolecular interactions with other components present in yeast and strongly favors intramolecular interactions within the fusion protein, reflecting steric differences in the estrogen receptor moiety of the fusion proteins. Figure 10 presents a simplified explanation of the steric differences observed. In the case of the wild-type estrogen receptor, agonist binding positions helix 12, and consequently the F domain, in an organized conformation for cofactor binding and transcription activation by AF-2. Mutating helix 12 (L540P) disorganizes the positioning of helix 12 and the F domain, causing interference. Interference is also caused by antagonists bound to the wild-type estrogen receptor, an effect that is partly relieved by deletion of the F domain. Helix 10 mutations (at 508) disorganize the positioning of helix 12 and the F domain upon agonist binding yet permit, in part, an organized positioning of helix 12 and the F domain upon antagonist binding, leading to activation (Fig. 8A). Deletion of the F domain relieves interference in all cases.

Antihormones are much larger molecules than hormones and probably interrupt the relatively compact LBD structure that would form around bound hormones (34,43). We reason that the antihormone-induced conformation of the EBD interferes with the FLP reaction, as it does with cofactor binding and transcription activation (AF-2) in the native receptor. We favor a model based on an organized positioning of helix 12 and the F domain by agonist binding. Previous work on ligand binding by nuclear receptors has implicated the correct positioning of helix 12 in agonist action (6,9,15,21,34). Our work extends these implications to demonstrate that the different positions of helix 12 adopted upon agonist or antagonist binding can have a dominant effect in a functional assay. Our work also highlights a role for the F domain in the differences between agonist and antagonist action.

Since AF-2 activity is not transcriptionally measurable in yeast, this presents a yeast assay for discrimination between hormones and antihormones at a structural level. For example, a compound which induces recombination with the 251 form but not the 304 form likely has antihormone properties and can be tested in full-size ER assays for confirmation.

### **Inversion mutations of other nuclear receptors.**

Several studies based on transcriptional assays have identified mutations in ER, and other steroid receptors, that convert antagonists into agonists (18,21,26,27,42). All of these mutations occur near to or in helix 12 and can, in the context of the work presented here, be evaluated as conformational mutations that disorder helix 12 and reposition the F domain. In one study, the ER helix 12 mutation, L540Q, inverted transcriptional activity so that antagonists induced activity but estradiol did not. Deletion of the F domain from the L540Q mutant ER restored estradiol-induced, and diminished antagonist-induced, transcriptional activity (27). This indicates that, by this mutation, the F domain is differently positioned upon agonist and antagonist binding in a transcriptional activity assay. Work on the glucocorticoid receptor (GR) mutation, I747T which lies between helices 11 and 12, documents a complementary observation of a difference between near normal binding of agonists and impaired transcriptional activity due to, we suggest, altered helix 12 position and F domain interference (35). We also suggest that different positioning of the F domain will differently affect co-factor binding. By analogy to the local steric effects on FLP recombination, we reason that a mispositioned helix 12 and F domain can interfere with local protein/protein interactions relevant to steroid receptor action. Of particular interest are interactions with steroid receptor transcriptional cofactors. Recent work has indicated that a variety of nuclear receptor cofactors exist (16 and references within; 15). It may be possible that different agonists and antagonists, by inducing different helix 12 and F domain positions, differently influence cofactor interactions. By this means, selectivity among cofactor binding directed by different ligands may be achieved. We also note that the same considerations of local protein/protein interactions may also influence receptor homo- or heterodimerization. Further work is required to establish the degree to which these speculations describe the transcriptional activities of the steroid receptors.

**The F domain length is conserved.**

The F domain may not be as inert for steroid receptor function as previously thought. It has not been included in any x-ray structures so far, for technical reasons, and yet abuts helix 12, important for ligand activated AF-2 function (9,15). An examination of F domains from all available steroid receptor sequences revealed a striking observation - namely, the lengths of the F domains have been largely conserved (Table IV). ER $\alpha$  has the longest F domain, 49 amino acids in all known higher vertebrates. Fish ER $\alpha$  F domains differ somewhat in that they are usually even longer, up to 83 a.a. All three known ER $\beta$  have F domains of 33 amino acids in length. Remarkably, all other steroid receptors have F domains of 18 amino acids in length, with one exception (trout glucocorticoid receptor has 24). Since there is little sequence conservation, even within a single receptor type, the conservation of F domain lengths is very unlikely to be coincidental. We reason that there are functional constraints that bear upon the F domain and that one of these constraints, as identified here, is the organized positioning of the F domain upon agonist binding.

**Hsp90 complex regulates LBD-fusion proteins.**

Current models to explain LBD regulation of proteins to which they are fused invoke a primary role for the Hsp90 complex (32). The Hsp90 complex is ubiquitous and abundant, and possesses chaperonin activity (33). Further recent evidence that the steroid receptor LBDs are associated with this complex in the unliganded state has come from genetic experiments with yeast (5,17,28). Fusion of an LBD onto a heterologous protein is believed to direct the fusion protein to associate with the Hsp90 complex (36). Binding of agonists promotes LBD release from the complex, thus derepressing the fusion protein functions. Whether all antagonists serve to release LBDs from the Hsp90 complex to the same extent remains unclear.

### Materials and Methods

**Strains and chemicals.** The *S. cerevisiae* strain used for these experiments (*MAT a*, *leu2-3,112*, *his3-11,15*, *ura3-52*, *trp1-1::(TRP1,URA3,SUP11)*, *ade2-1ochre*, *can1-100*) was derived from RS453 (R. Serrano, Valencia, Spain) by integrating the target of recombination (Fig. 1A) at the *trp1* locus. Transformation of yeast by the standard lithium acetate method was performed as described (2). Transformed yeasts were grown and maintained with selection for leucine and tryptophan in glucose or galactose supplemented synthetic media from BIO 101, Inc. The hormones and antihormones were purchased from Sigma, except 4-hydroxytamoxifen (Research Biochemicals International), and ICI 182,780 (a gift from Dr. A. Wakeling, Zeneca Pharmaceuticals).

**Southern assays and ligand titration experiments.** Transformed yeast, containing the GAL10 promoter, FLP-ER gene on pRS315 (38), were grown in synthetic glucose medium lacking leucine and tryptophan to OD<sub>600</sub> = 1.5. Equal volumes of cultures were collected and resuspended in medium containing 2% galactose with or without ligands, which was dissolved in ethanol as a 1000 or 10,000 fold stock solution. The "no hormone" samples received an equal volume of ethanol. Cells were collected at times noted and DNA was prepared by standard procedures using a zymolyase 20T (ICN) incubation, SDS lysis, followed by potassium acetate precipitation as described (2). About 10 µg of DNA per lane was digested with PstI and loaded on 0.7% gels in 1x TAE buffer. Gels were treated with 0.25M HCl for 10 min, 0.4M NaOH for 2x 30 min, 20x SSC for 30 min, and then blotted to Qiagen nylon plus filters with 20x SSC. After baking the filter at 80°C for 2 hr, they were probed at 72°C with a riboprobe, made from the 1.2 kb Scal-BsiWI fragment of the *E. coli* LacZ gene, in a buffer containing 250 mM sodium phosphate pH 7.2, 7% SDS, and 1 mM EDTA. Washes were performed in 25 mM sodium phosphate pH 7.2, 1% SDS and 1 mM EDTA at 72°C. Recombination was calculated as a ratio of [counts in the recombined band/(counts in recombined + unrecombined bands)] and was therefore not affected by minor variations in the amount of DNA loaded.

**Ligand binding assay.** Ligand binding experiments were performed to measure estradiol, 4-hydroxytamoxifen, raloxifene and ICI 182,780 binding by the various FLP-ER fusion proteins. The protein extracts were made from FLP-ER transformed yeast, grown in galactose

without hormones to  $OD_{600} = 1$ . The resuspended yeast pellets were lysed using a glass bead procedure (2) in a buffer containing 20mM Tris, pH 7.9, 10mM  $MgCl_2$ , 1mM EDTA, 5% glycerol, 1mM DTT, 420mM KCl and protease inhibitors. The ligand binding experiments were done in 300  $\mu$ l volume with a fixed concentration (1nM) of radiolabelled  $^3H$ -estradiol (84 Ci/mmol; Dupont NEN), which was pre-mixed in the respective tubes with zero or increasing amounts of unlabelled ligand (1nM to 1000nM). The binding was at 4°C for 16-18 hours in buffer (PMMG) containing 8.5mM  $Na_2HPO_4$ , 1.5mM  $KH_2PO_4$ , pH 7.5, 10mM sodium molybdate, 2mM monothioglycerol, 20% glycerol and 1 mg/ml protein extract. After the binding incubation, unbound label was absorbed by adding 300 $\mu$ l of DCC (0.5% charcoal Norit-A, 0.05% dextran T70) in PMMG buffer for 15 min at 4°C and then centrifuging at 12,000 rpm for 5 min. Equal volumes of supernatant were quantified by liquid scintillation counting. Binding values ( $IC_{50}$ ) are expressed as the amount of unlabelled ligand competitor needed to reduce to 50% the  $^3H$ -estradiol bound in the absence of unlabelled steroid.

**Color plate assay.** Yeast containing the integrated SUP11 recombination substrate (Fig. 1B) were transformed by plasmids containing the various FLP-ER genes. Cultures were grown in glucose with leucine selection and then plated at high density on synthetic galactose plates lacking leucine and tryptophan. A 2  $\mu$ l drop of ethanol containing each ligand was placed on the plate, as shown (Fig. 2, 6B). An adenine marker in yeast gives a color phenotype (red) if the target gene has been deleted by the recombinase. The plates were grown at 30°C for 4 days to maximize red color formation.

### **Generation of mutated FLP-ER libraries by codon substitution mutagenesis (CSM)**

CSM was performed as described (8) on amino acids of the human estrogen receptor (outlined in last year's report).

## CONCLUSIONS

The properties of estrogen ligand-inducible recombination mediated by FLP-EBD fusion proteins show that all ligands activate FLP-EBD fusion proteins with a D domain, yet without, antihormone binding forms an EBD conformation which blocks recombination by a steric mechanism, emanating from a misaligned helix 12 and F domain. This presents a simple assay to predict hormone vs. antihormone activity of a compound in yeast, as well as a way to screen for functional interactions with amino acids defining hormones vs. antihormones. We have used codon substitution mutagenesis (CSM) to generate mutagenized libraries. Estrogen induced changes in yeast colony color has been used as a simple method to detect ligand binding, and to measure its relative hormone and antihormone character. There has been a change of focus from exclusively ligand binding changes to include those mutations which cause functional differences between agonists/ antagonists. We have addressed this with work demonstrating helix 12 and F domain importance.

Future experiments will include:

1. Mammalian cell culture experiments to test transcription responses of these mutants in full-size native hER. The FLP-EBD fusions discussed here exhibit almost identical properties in mammalian cells as in yeast (data not shown).
2. screens of CSM libraries of EBD mutations already in progress. These libraries will be searched for mutations that alter responsiveness to known estrogen hormones and antihormones (Fig 1); for ones which increase responsiveness to ligands that are poor estrogens; and to define the repressive functions of the EBD, as exhibited by either constitutive recombinase activity or failure to respond to authentic estrogens. We have many such mutations to characterize already (Table III).
3. further detect and measure estrogenic activities, including work addressing estrogens of environmental importance and phytoestrogens.
4. continue direct ligand binding assays to measure ligand affinity changes with many of the mutations already generated.

Can amino acid determinants of ligand binding specificity in the estrogen receptor be found? Can the FLP-EBD fusion proteins define precisely the functional components of hormone versus antihormone action? We have made good progress toward understanding components of the LBD that participate in antihormone blockage of ER. Many of the

mutations we have studied have generated altered LBD structures, as opposed to ligand affinity changes. Others clearly have an affect on one or two ligands only. With several of the ligand specific mutations we have found, we wish to get a better understanding of how tamoxifen and raloxifene bring about tissue-specific effects. The long-term goal is to develop better therapeutic agents, stemming from more complete molecular information.

The following papers have resulted from the work so far; others are in preparation.

1. Stewart, A.F., Logie, C. and Nichols, M. (1996) Regulation of nuclear receptors by agonists and antagonists. *Curr Opin Endocrin & Diabetes* **3**: 397-402.
2. Nichols, M., Rientjes, J.M.J., Logie, C. and Stewart, A.F. (1997) FLP recombinase/ estrogen receptor fusion proteins require the receptor D domain to convey responsiveness to antagonists, but not agonists. *Mol. Endocrinol.* **11**: 950-961.
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## Figure Legends

**Figure 1. (A) Diagram of the FLP-EBD expression plasmid displaying its functional elements.** The gene for the FLP-ER LBD fusion protein is driven by a galactose promoter on a plasmid in yeast. **(B) Diagram of the FLP recombination deletion strategy.** Before recombination, the constitutively active ADH1 promoter expresses the URA3 selectable marker which lies between directly repeated FLP recombination targets (FRTs, shown as triangles). The polyadenylation signals (pA) after URA3 prevent LacZ expression. Also lying between the recombination targets is a SUP11 gene which is transcribed in the opposite direction, as depicted by the short arrow. Downstream of the second recombination target is the LacZ coding region. After recombination, the URA3/SUP11 region is excised and the LacZ gene juxtaposed to the ADH1 promoter. Recombination mediated alterations in cellular phenotype are displayed at the right of the diagram. Note that expression of the endogenous Ade2<sup>+</sup> gene relies on SUP11 expression. The diagram also outlines the Southern strategy employed. A 5.6 kb fragment is reduced by recombination to a 4 kb fragment when a probe from the LacZ gene is used. **(C) Structures of the ligands tested with the FLP-EBD.** The hormones are shown above the line, antihormones below.

**Figure 2. Color plate assay, comparing the effect of the D domain with activation by antagonists.** FLP-EBD recombination in a color plate assay in yeast reflects affinity and the concentration of a hormone placed on a lawn of unrecombined yeast cells. Yeast containing the SUP11 recombination substrate (Fig. 1) and either the WT251 (FLP-D/E/F) or WT304 (FLP-E/F) fusion proteins were plated at high density onto low adenine medium in a standard 9 cm petri dish. As is apparent, the D domain selectively alters ligand responsiveness. Deletion of the D domain (WT304) renders the fusion recombinase insensitive to any of the antihormones tested, as opposed to the FLP-EBD<sub>wt</sub> (WT251) control. An adenine marker in yeast gives a color phenotype (red) if the target gene has been deleted by the recombinase. A 1  $\mu$ l drop of ethanol containing the ligands were plated on the yeast lawn and grown for several days.

**Figure 3. Ligand binding and Western blots of FLP-EBD proteins.** The top panel shows the results of *in vitro* ligand binding competitions with the FLP-EBD<sub>251</sub> and the FLP-EBD<sub>304</sub> forms. Labelled <sup>3</sup>H-estradiol (1nM) was bound without or with increasing amounts of unlabelled estradiol (E2) or 4-hydroxytamoxifen (Z-OHT). The 100% binding points are determined in the absence of added cold ligand, minus background (marked NL). The lower panel shows the results of western blot experiments, using an antibody to the C-terminal, aa 576-595 of ER. The natural human ER is present at 66kDa in MCF-7 cells. FLP recombinase/ ER fusion proteins are larger; the WT<sub>251</sub> fusion form is 88kDa and the WT<sub>304</sub> form is 82kDa. Two mutated forms of the EBD (251-17, 304-17) do not change the steady-state amount or the size of protein recovered from yeast.

**Figure 4. Schematic representation of various forms of the estrogen receptor fused to FLP, with ligand affinities for each.** In these constructs, the D domain starts with hER amino acid 251, the E domain with a.a. 304 and the F domain deletion omits a.a. 552-595. Positions of the helix 10 mutation (L508R) and the helix 12 mutation (L540P) near the C-terminal end of the E domain are also shown as asterisks. IC<sub>50</sub>, the amount of cold ligand competitor needed to reduce 1 nM <sup>3</sup>H-estradiol bound in the absence of cold steroid to 50% is shown for each of the FLP-ER forms and ligands. None of the mutations or deletions shown significantly affect binding by the ligands. E2-estradiol, Z-OHT- 4-hydroxytamoxifen, RAL- raloxifene, ICI 182,780.

**Figure 5. The activity of FLP-ER fusion recombinases is affected by the presence of the D and F domains, as well as helix 12 integrity.** The figure shows southern blots used to measure recombinase activity in ligand titration experiments with either estradiol (E2) from 10<sup>-6</sup> to 10<sup>-10</sup> M or 4-hydroxytamoxifen (Z-OHT) from 10<sup>-5</sup> to 10<sup>-9</sup> M. Control samples were cultured in glucose (gl, first lane) or galactose without ligands (-). Panel A shows experiments with FLP-ERs without the D domain; with (FLP-E/F) or without (FLP-E) the F domain; or including the L540P mutation with [FLP-E(L540P)/F] or without [FLP-E(L540P)] the F domain. Panel B shows the equivalent FLP-ERs which contain the D domain.

**Figure 6. Isolation of mutations which show altered specificity of ligand induced recombination.** In step 1, libraries of FLP-ER mutagenized between ER a.a.s 506-527 were grown in the presence of estradiol to activate FLP-ER recombination and in the absence of uracil to select against recombination. Thereby FLP-ER mutants not activated by estradiol were enriched. In step 2, the surviving cells were collected and grown in the presence of a second ligand (Z-OHT or raloxifene) in the presence of uracil to permit recombination and then screened on plates for red colonies indicating recombination (Fig. 1B). Plasmids containing candidate mutations were rescreened to verify estradiol resistance and antagonist inducibility.

**(B) Colony color plate assay to evaluate ligand responsiveness of FLP-ERs.** The plasmids were retested in the parent strain to confirm differential inducibility by a set of ligands, as shown, in plate assays. E2- estradiol, Z-OHT- 4-hydroxytamoxifen, RAL- raloxifene, DES- diethylstilbestrol, TAM- tamoxifen, HEX- hexestrol. FLP-E/F is activated only by agonists (E2, HEX, DES) and a representative helix 10 mutation (FLP-E(L508E)/F) is activated only by antagonists (Z-OHT, RAL, TAM).

**(C) Southern blots used to measure recombinase activity in ligand titration experiments,** as in Figure 5. A comparison of the relative ligand activities for mutation LBDs from our screen (L508E) and a previously identified mutation (G521R) with agonist/antagonist reversed activity. Both types of FLP-ER construct, with and without the D domain, are compared, and confirm the inversion phenotype.

**Figure 7. Inversion mutations show activation by antihormones and not by hormones.** Two additional mutations which resulted from the screen outlined in Fig. 6, are tested for ligand responsiveness by Southern blotting. Both isolates, 13 (L508D,L509R) and 23 (L508K) show activation by antihormones Z-OHT and Raloxifene and resistance to estradiol, opposite to the wild-type FLP-EBD response.

**Figure 8. Helix 10 inversion mutations are not activated by agonists due to F domain interference.** The figure shows southern blots used to measure recombinase activity in ligand titration experiments as in Figure 5. Panel A shows experiments with the L508R inversion mutant without the D domain; with [FLP-E(L508R)/F] or without [FLP-E(L508R)] the F domain; or including the L540P mutation with [FLP-E(L508R,L540P)/F] or without [FLP-E(L508R,L540P)] the F

domain. Panel B shows the equivalent FLP-ERs which contain the D domain, as additional controls for the ligand binding properties of these mutations, and to illustrate the proximity dependence of F domain interference.

**Figure 9. Plate assays show that a disruption mutation of helix 12 causes the singular loss of raloxifene activity.** The plates shown have ligands placed as in Fig. 2 and Fig. 6B. Deletion of the F domain increases activity slightly, especially for the L540P, helix 12 disrupting mutation (right-hand panel).

**Figure 10. Schematic diagram of various agonist and antagonist bound forms of the FLP-ER showing positioning of helix 12 and the F domain which alters interference with the FLP recombinase activity.** Helix 10 is signified by the thick bar inside the ligand binding domain (enclosed region) and helix 12 and the F domain are depicted outside the enclosed region as a thick bar and thin line, respectively.

**Figure 11. The protein sequence of the C, D, E, and F domains of human estrogen receptor.** The fusion points used here for the FLP chimeras are signalled by the arrows at 251 (FLP-D/E/F) or 304 (FLP-E/F). The structural elements of the hormone binding domain, deduced from sequence alignments and the known RAR structure (34,44), are mapped onto the sequence of the human ER. Boxes outline  $\alpha$ -helices (H1 to H12) and arrows mark  $\beta$ -sheets (S1, S2). The positions of inversion mutations found in helix 10 (L508R), disruption mutations of helix 12 (L540P), and the sizable F domain (49 aa) are also marked.

**Figure 12. Altered FLP-EBD recombination in a plate assay in yeast,** as described in Fig. 2. Here a mutation causes increased response to agonist ligands and also a specific response to raloxifene. Whether the change includes ligand affinity is not yet tested.

**Table I.** Mutations recovered by selection for agonist insensitivity/antagonist activation are shown underneath the amino acid sequence of the C-terminus of the ER ligand binding domain, E. The positions of helices 10, 11 and 12 are indicated.

helix 10					helix 11					helix 12 (AF-2)														
510					520					530					540					550				
HQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVPLYDLLLEMLDAHRLH																								
E																								
R																								
K																								
Q																								
I					E																			
R															L									
DR																								
D															K									
VK																								
H															K									
RD															Y									
RH					G																			
SA					V																			
VGS																								
RA					T																			
A					T					D														
VQ					A					C														
HQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVPLYDLLLEMLDAHRLH																								
510					520					530					540					550				



Table II.

## Human Estrogen Receptor - LBD

## Wild-Type

helix 10	helix 11	helix 12 (AF-2)
510	520	530
540	550	
<u>HQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVPLYDLLLEMLDAHRLH</u>		
G		
I		
V		
G		
A		
A		
A	T	
	G	
	Q	
	V	
S		
G EC		
<u>HQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVPLYDLLLEMLDAHRLH</u>		
510	520	530
540	550	
helix 10	helix 11	helix 12 (AF-2)

## weak wt

helix 10	helix 11	helix 12 (AF-2)
510	520	530
540	550	
<u>HQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVPLYDLLLEMLDAHRLH</u>		
R		
NV		
N	P	
	Q	
	V	
S	N	
PG		
P	P	
	CL	
	G	
	S RG	
<u>HQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVPLYDLLLEMLDAHRLH</u>		
510	520	530
540	550	
helix 10	helix 11	helix 12 (AF-2)

## WT +

helix 10	helix 11	helix 12 (AF-2)
510	520	530
540	550	
<u>HQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVPLYDLLLEMLDAHRLH</u>		
S		
E N		
H L T		
N	I	

Table II (cont).

## altered specificity

helix 10	helix 11	helix 12 (AF-2)
510	520	530
540	550	
<u>HQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVPLYDLLLEMLDAHRLH</u>		
G		
R G		
GT		
N		Y
		Y
	A R	
	T IY	

## Inactive

helix 10	helix 11	helix 12 (AF-2)
510	520	530
540	550	
<u>HQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVPLYDLLLEMLDAHRLH</u>		
MG		
KA		
	R	
R	I	
S R		
T	L	
RDR		
D	G	
G C	A	
G P	L	
GI	G	
E R	C	
	TC R	
A	T Y	
	A FT	
RAA		
Q WP A		
Term		
Term		
Term		
fs	Term	
fs	Term	
fs	Term	
<u>HQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVPLYDLLLEMLDAHRLH</u>		
510	520	530
540	550	

**Table III. EBD mutations.**

C 530 L  
 M 528 L  
 K 529 C  
 Term 527 am  
 fs 524, Term 526  
 fs 529 (term 538)  
 K 529 I  
 K 529 T  
  
 L 511 K  
 L 508 W, L 509 S, I 510 A  
 R 515 G  
 L 511 A  
 L 509 F, M 517 E, K 520 G, G 521 V  
 H 516 D, H 524 R, L 525 A, S 527 R  
  
 G 521 R  
 L 507 G, L 511 G, K 529 N, C 530 A  
 L 511 P  
 K 531 R  
 I 510 A, R 515 T  
 L 508 A, L 509 G, I 514 L, H 516 R  
 L 508 T, M 517 D  
 L 511 R, H 516 A  
 L 507 I, L 508 K, I 510 A, H 513 Y  
 I 510 P, L 511 P  
 Q 506 D, L 508 Y, I 510 L, L 511 R  
 L 509 S, I 510 A, L 511 D, R 515 G  
 fs 513, Term 518  
 fs 515 ( $\Delta$  13 bp)  
  
 K 520 T, G 521 C, E 523 R  
 H 516 V, M 517 P, M 522 A, L 525 T, Y 526 G  
 L 511 S  
 I 510 S, L 511 Term am (H 513 E)  
 Q 506 T, L 507 P, L 508 Term op  
 L 509 V  
 N 519 Term am  
  
 Q 314 P, Term 315 op  
  
 fs 425, Term 457  
  
 L507V, L508Q, L509W, I510S, L511 Term  
 L 508 Term op,  $\Delta$  23 bp  
 I 510 G  
 I 510 E, H 513 N  
 L 509 K, I 510 R, fs 519  $\Delta$  4, Term op 527  
 L 509 R  
 Q 506 G, I 510 E, L 511 C  
 D H516, D M517 ( $\Delta$  6 bp)  
 I 514 Q  
 L 508 Term op,  $\Delta$  23 bp  
 L 509 D, R 515 K  
 L 508 R, L 509 H, L 511 G

L 508 W, I 510 T, fs 511, Term 517  
 I 510 P, L 511 S,  $\Delta$  15 bp, M517 wt>  
 L 508 S, L 509 G, I 510 S, fs 511, 517 Term  
 fs 511, Term 519 och  
 L 508 E, I 510 R, R 515 C  
 L 507 K, L 508 A  
 L 507 A, L 509 T, S 512 D  
 Q 506 G, L 508 P, I 514 L  
 L 507 N, L 508 V  
 I 510 N, M 517 P  
 I 510 S, M 517 V  
 L 511 P, R 515 P  
 L 508 P, I 510 A, L 511 Term op  
 L 507 G, L 511 C, R 515 A  
 L 509 S, L 511 R  
 L 509 R, S 512 L, H 513 Term op  
 Q 506 L, L508H, L509R, I510E, M517 Term  
 R 515 G  
 L 508 H, I 510 L, I 514 T  
 I 510 A  
 S 512 G, M 522 L  
 G 521 Q, M 522 L, E 523 D, Y 526 G  
  
 L 507 M, L 508 G  
 H 524 Y  
 R 515 C, H 516 L  
 I 510 R  
 R 515 V  
 I 514 Q  
 L 507 I  
 L 508 R  
 L 508 R, L 540 P  
 L 507 G  
 L 507 Term  
  
 L 507 G, L 508 T  
 L 509 N, H 524 Y  
 L 508 I, S 512 E  
  
 L 508 D, L 509 R  
 L 509 D, R 515 K  
 L 507 V, L 508 K  
 L 508 R, L 509 D, M 517 Y  
 L 508 R, L 509 C, S 512 Q  
 L 508 R, L 509 A, L 511 T  
 L 507 V, L 508 Q, L 511 A, R 515 C  
  
 Y 526 G  
 L 508 P, L 525 P  
 H 524 S, Y 526 R, S 527 G  
 H 516 D, N 519 F, K 520 T  
 N 519 T, E 523 I, H 524 Y  
  
 R 515 Q  
 G 521 A, E 523 R  
 I 510 P, L 511 G

**Table III. (continued)**

L 508 K  
 L 508 T, R 515 L  
 L 507 D, I 514 G  
 L 508 G, L 509 I, R 515 G  
 L 508 S, L 509 A, S 512 V  
 L 507 V, L 508 G, L 509 S  
 L 508 H, I 510 L, I 514 T  
 L 508 D, L 509 R  
 L 509 R, D 510-511  
 L 511 R, M 517 I  
 L 508 R, M 517 L  
 L 508 H, R 515 K  
 I 510 S, S 518 N  
 L 509 Q, L 511 W, S 512 P, R 515 A  
 I 510 D,  $\Delta$  511-512, R 515 K  
 H 524 Y  
 L 509 R, S 512 G  
 D509, H 513 T, M 517 Y

L 508 K  
 L 508 G, L 509 I, R 515 G  
 L 508 E  
 L 508 H, I 510 L, I 514 T  
 L 508 Q  
 L 508 H, R 515 K  
 L 508 R, M 517 L  
 L 509 R, S 512 G  
 L 509 R, I 510 D, L 511 R  
 L 509 N, M 517 I  
 L 509 V

Term 406 och  
 S 395 P  
 A 382 K, L 384 P, I 386 Term op  
 H 398 L, fs 401, Term 405 och

H 398 Term amb  
 K 401 S, L 402 Q, F 404 W, P406L, N407M,  
 D 411 R, R 412 N, N 413 Q, Q 414 R

L 408 D, N 413 F, G 415 P, G 420 L  
 R 412 A, N 413 R, C 417 Q, V 418 L, E 419 E  
 419 A, G 420 A, M 421 S  
 F 461 Q, fs 463, Term 518  
 L 453 F, L 466 R  
 G 457 H  
 N 455 F, V 458 H, Y 459 P, T 460 F, S 464 V  
 M 427 K, S 432 C, R 436 Term amb  
 S 433 L, R 434 P, F 435 Y, R 436 G  
 A 430 E, Q 441 L

S 464 C  
 F 461 A  
 S 433 N  
 fs 431 ( $\Delta$ 1)

P 399 S, K 401 G, L 402 Term och  
 G 420 N  
 N 413 I, G 420 L, M 421 K  
 N 413 D, fs 416  
 D 411 Y, M 421 N  
 N 407 D, D 411 P, E 419 W, G 420 K  
 P 406 G, D 411 T, R 412 T, N 413 Q, Q 414 R  
 P 399 G, G 400 R, L 403 K, L410P, D411L,  
 R 412 N, N 413 Q, Q 414 R

C 381 G, A 382 E  
 E 470 Q  
 L 466 A  
 Y 459 I, fs 464 ( $\Delta$ 1)  
 M 437 L  
 A 382 V, M 388 F, W 393 A  
 L 391 P  
 C 381 fs, 384 Term amb  
 A 382 fs, 391 Term opal  
 P 399 G, L 402 fs, Term > 416  
 K 401 G, L 409 A, R 412 N, N 413 Q, Q 414 R

L 453 G, G 457 Q  
 L 454 P, V458L,  $\Delta$ 461-464 (FLSS), T465P  
 S 456 D, G 457 V  
 I 451 T, Y 459 C, S 464 L  
 N 455 P, F 461 R, S 464 R, T 465 H, L 466 V  
 N 455 R  
 S 456 P, S 464 C  
 I 451 A, L 454 P, G 457 N, T 460 L  
 N 455 S, T 460 H, S 464 F

L 428 V, L 429 T, F 435 G  
 R 436 G, M 437 D  
 T 431 R  
 M 438 G  
 M 427 fs, M 437 Term opal  
 L 428 G, L 429 E, M 437 S

L 508 D  
 fs 432, Term 454 ochre  
 S 432 V, S 433 T  
 M 427 T  
 M 437 H  
 L 429 Q  
 M 517 C, M 522 G, H 524 L, S 527 E

M 427 R, L 428 G  
 S 456 F, Y 459 P, L 462 K  
 I 451 A, L 454 P, G 457 N, T 460 L  
 N 455 S, Y 459 P, L 462 S, S 463 N  
 S 456 K, Y 459 S  
 N 455 S, Y 459 P, L 462 S, S 463 N  
 I 452 N, S 456 P, S 463 F

C 381 E, A 382 Term amb, I 389 A, G 390  
 P, W 393 G

**Table IV. The F domain amino acid length, but not sequence, is conserved.** Listed are the number of amino acids following the last one of the ligand binding domain helix 12, as defined by the alignment in Wurtz et al., 1996 (44).

<b>Receptor</b>	<b>Species</b>	<b>Length (a. a.s)</b>
Estrogen $\alpha$ :	Human	49
	Mouse	49
	Rat	49
	Chicken	49
	Pig	49
	Japanese eel	47
	Rainbow trout	63
	Atlantic salmon	63
	Medaka fish	70
	O. aureas (fish)	83
Estrogen $\beta$ :	Human	33
	Mouse	33
	Rat	33
Progesterone:	Human	18
	Mouse	18
	Chicken	18
	Rabbit	18
Androgen:	Human	18
	Mouse	18
	Rat	18
	Rabbit	18
Mineralo-corticoid:	Human	18
	Rat	18
	Tree shrew	18
	Xenopus	18
Gluco-corticoid:	Human	18
	Mouse	18
	Rat	18
	Xenopus	18
	Guinea pig	18
	Rainbow trout	24

Figure 1.

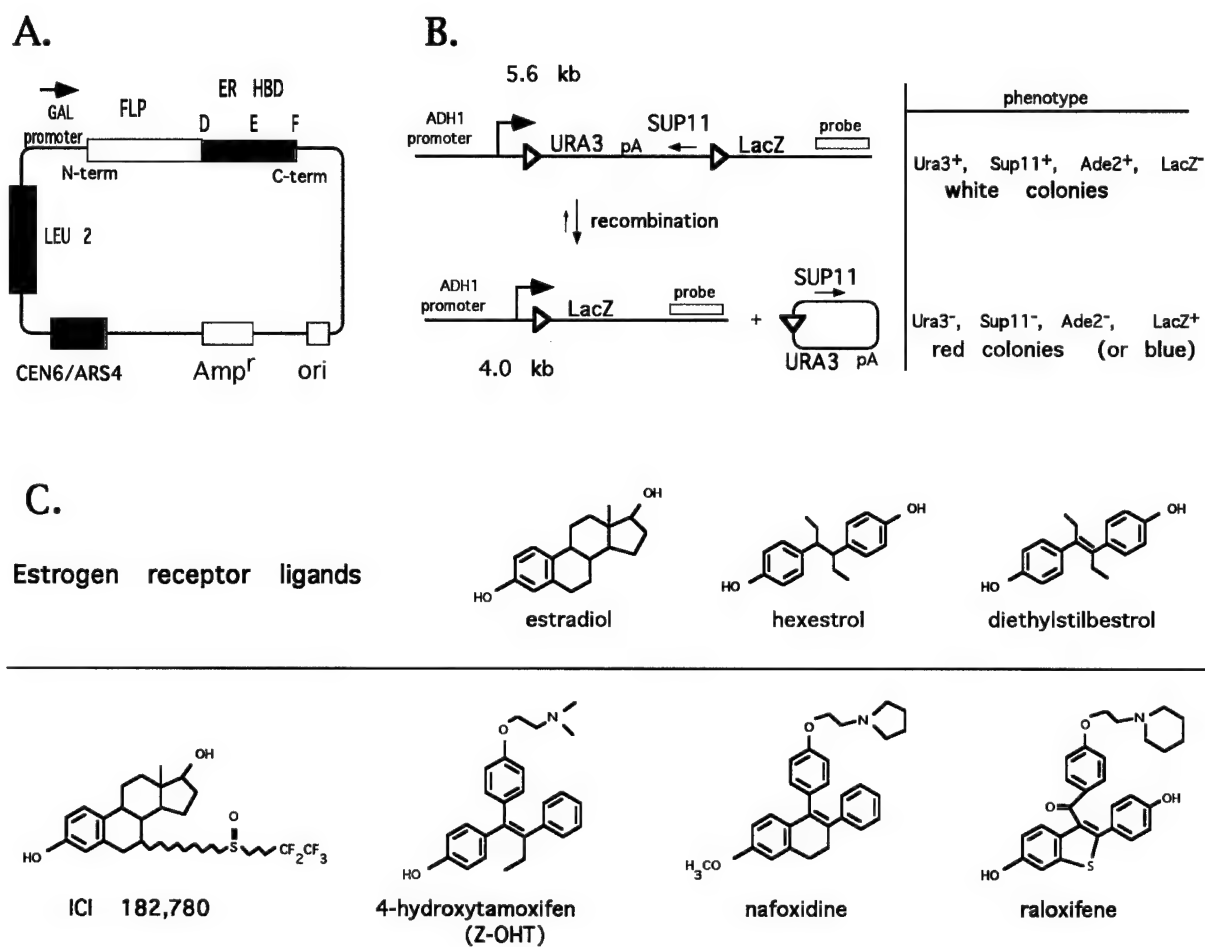


Figure 2.

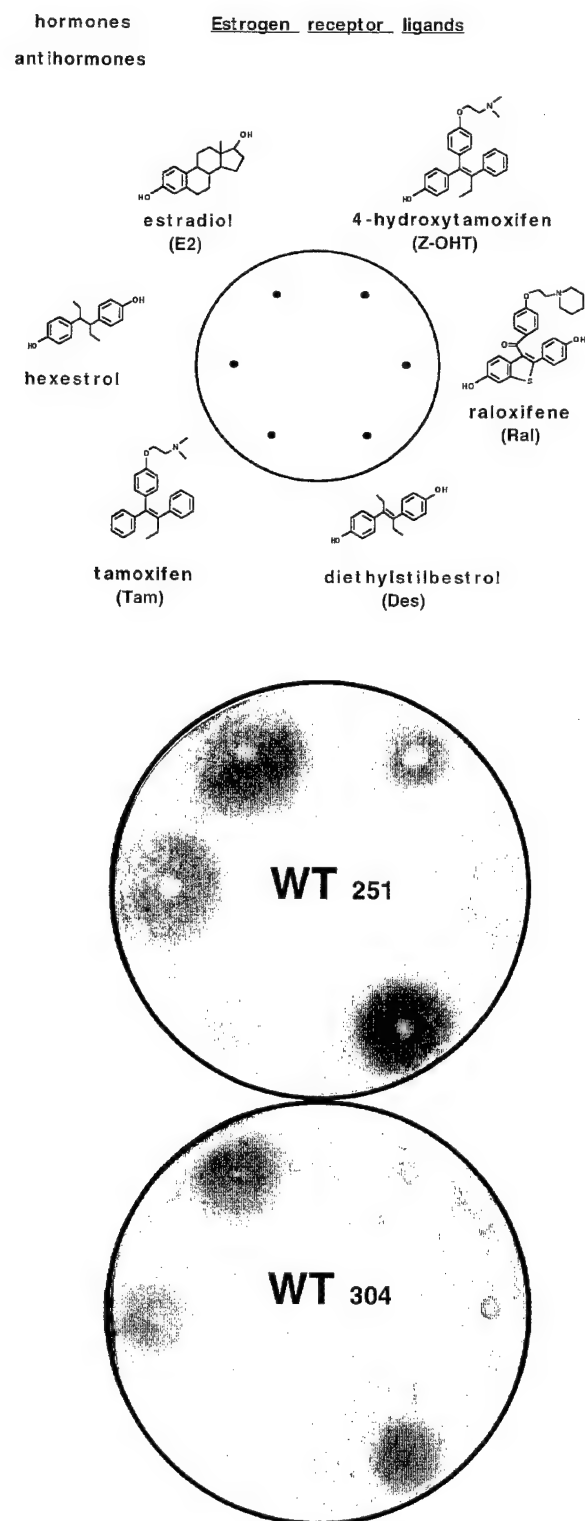
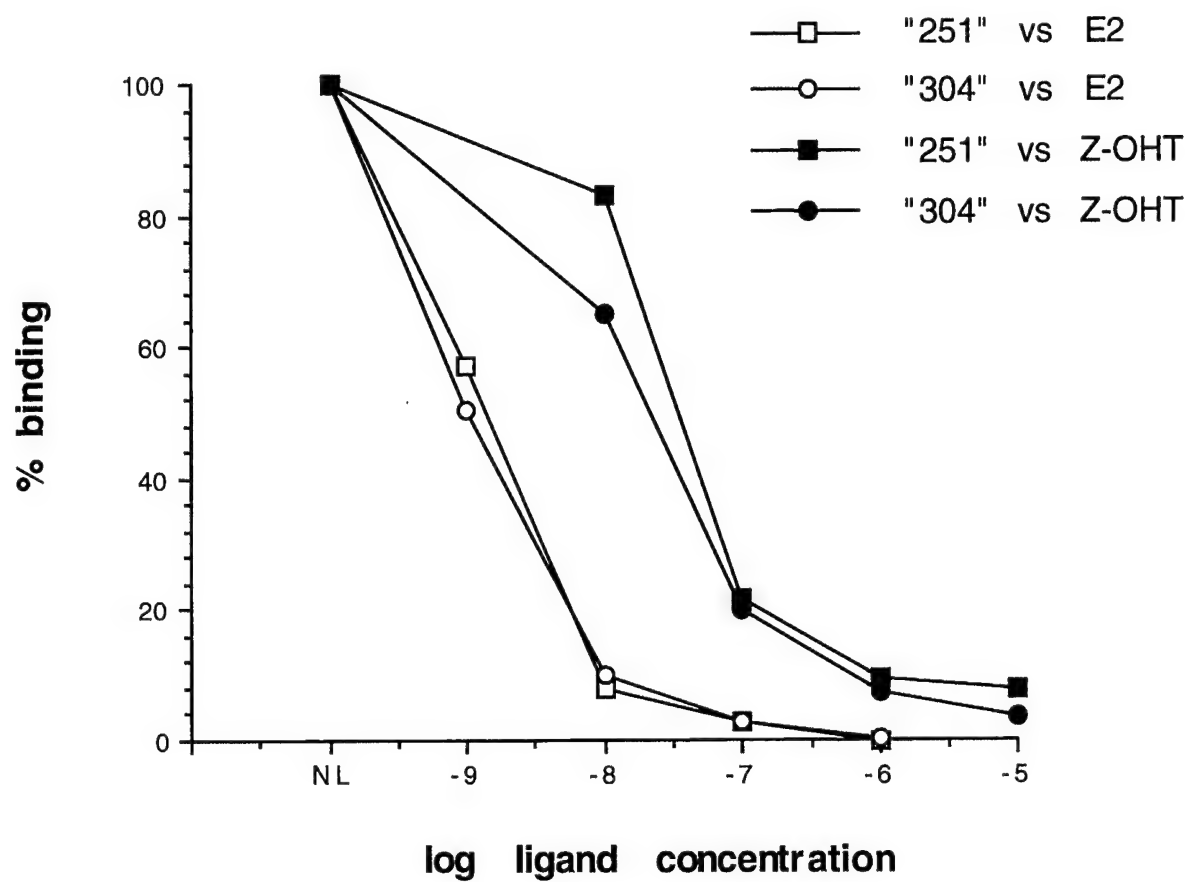


Figure 3.



$\alpha$  ER (aa 576-595)

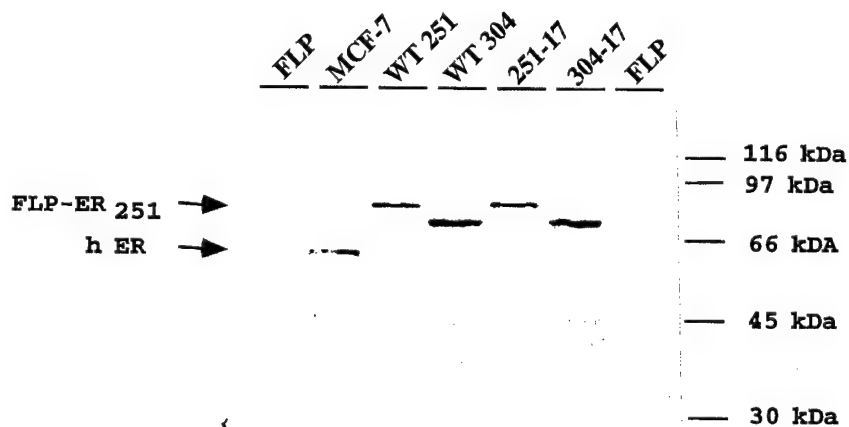




Figure 4.


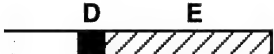
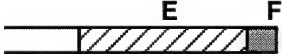



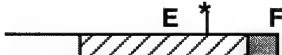

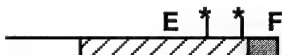

		IC <sub>50</sub> (nM)			
		E2	ZOHT	RAL	ICI
	FLP-D/ E/ F	0.6	1.6	1.5	1.5
	FLP-D/ E	0.8	2.1	1.8	1.9
	FLP-E/ F	0.8	2.0	1.5	2.3
	FLP-E	0.5	1.3	1.2	1.7
	FLP-E( L540P)/ F	0.8	0.6	0.7	1.0
	FLP-E( L540P)	0.9	0.6	1.0	0.7
	FLP-E( L508R)/ F	0.6	1.4	2.5	2.0
	FLP-E( L508R)	0.7	0.8	2.2	1.3
	FLP-E( L508R, L540P)/ F	0.8	0.5	0.8	0.6
	FLP-E( L508R, L540P)	0.6	0.5	0.7	0.6

Figure 5

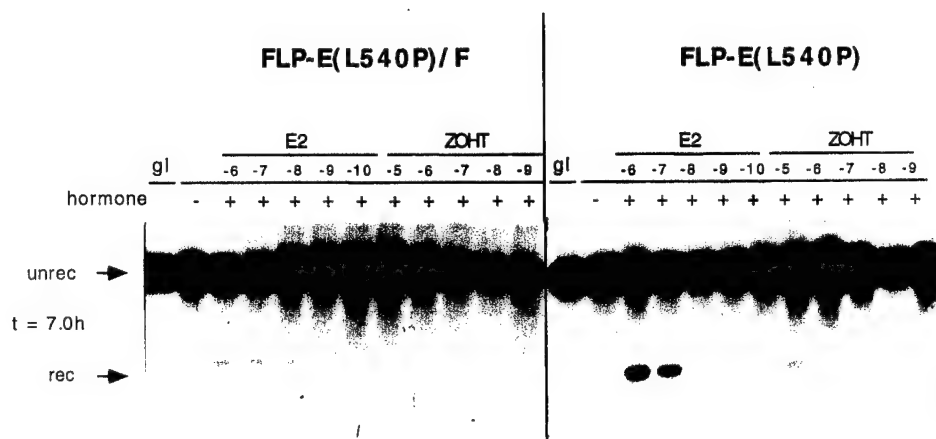
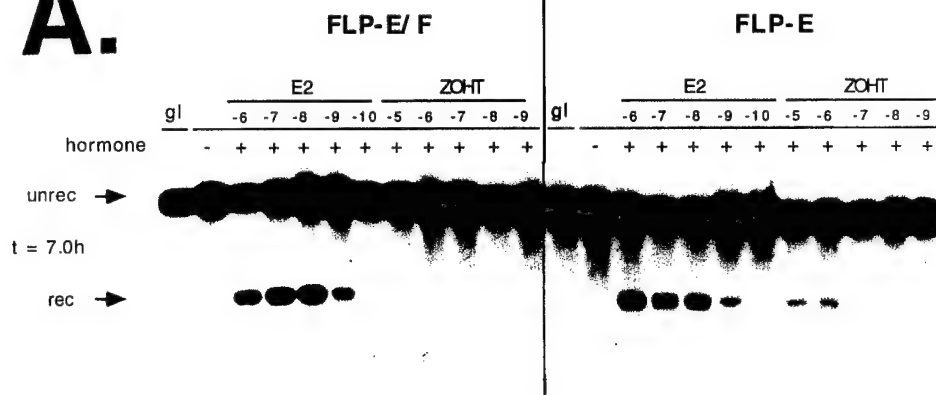
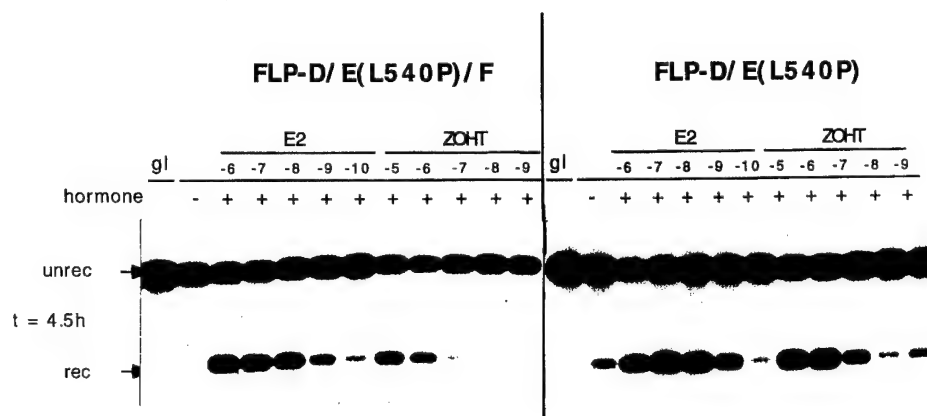
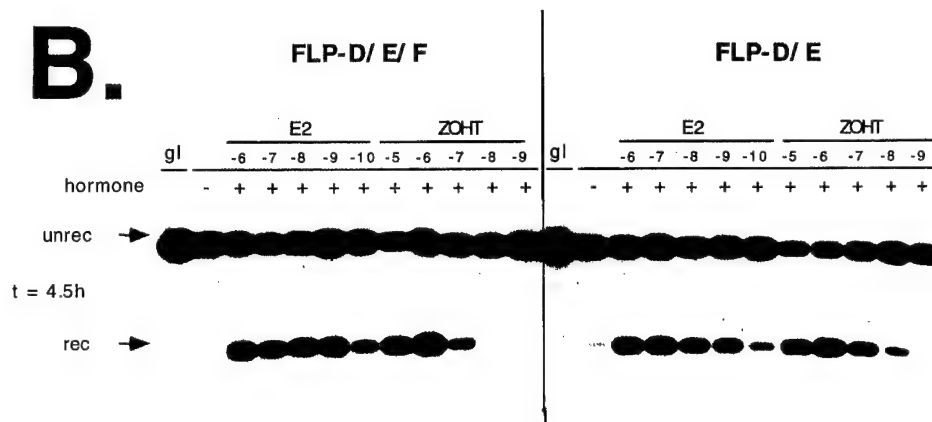
**A.****B.**

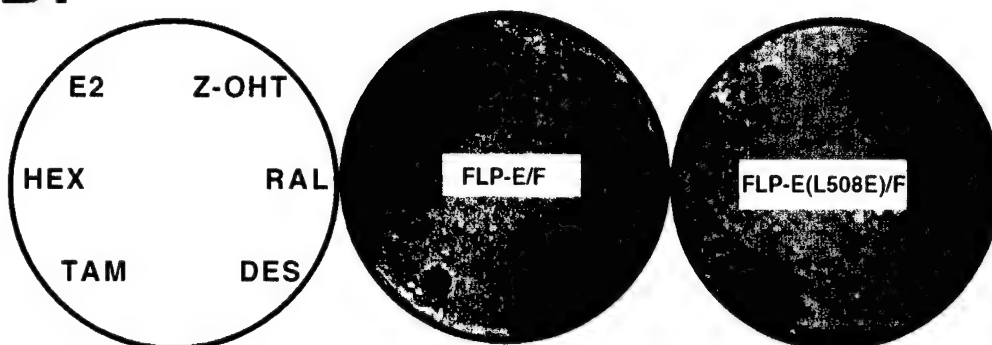
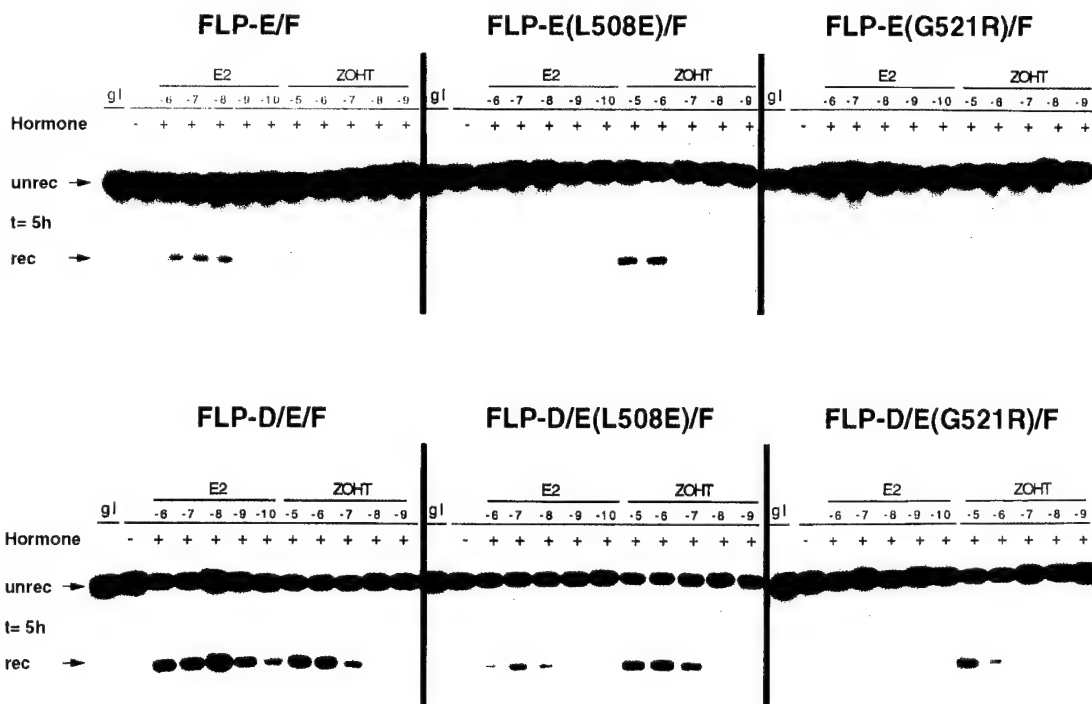
Figure 6.

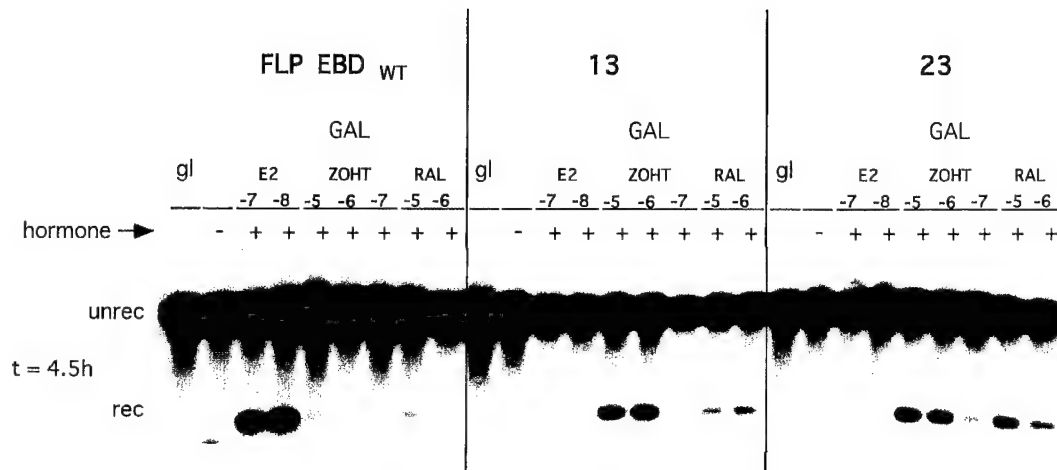
**A.**

Step 1. Select against recombination in the presence of E2

Step 2. Screen for recombination in the presence of Z-OHT

Step 3. Isolate the FLP-ER plasmid and retransform for plate assay of ligand activity

**B.****C.**



**FLP-E( L508 R) / F**

**FLP-E( L508 R)**

**FLP-E(L508R,L540P)/F**

**FLP-E(L508R,L540P)**

# B.

**FLP-D/ E( L508R) / F**

**FLP-D/ E( L508R)**

**FLP-D/ E( L508R,L540P)/ F**

**FLP-D/ E( L508 R,L540 P)**

Figure 1																		
		E2					ZOHT											
		gl	-6	-7	-8	-9	-10	-5	-6	-7	-8	-9	gl	-6	-7	-8	-9	
hormone		-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	
unrec		→	[band intensity across lanes]															
t = 4.5h																		
rec		→	[band intensity across lanes]															

Figure 9.

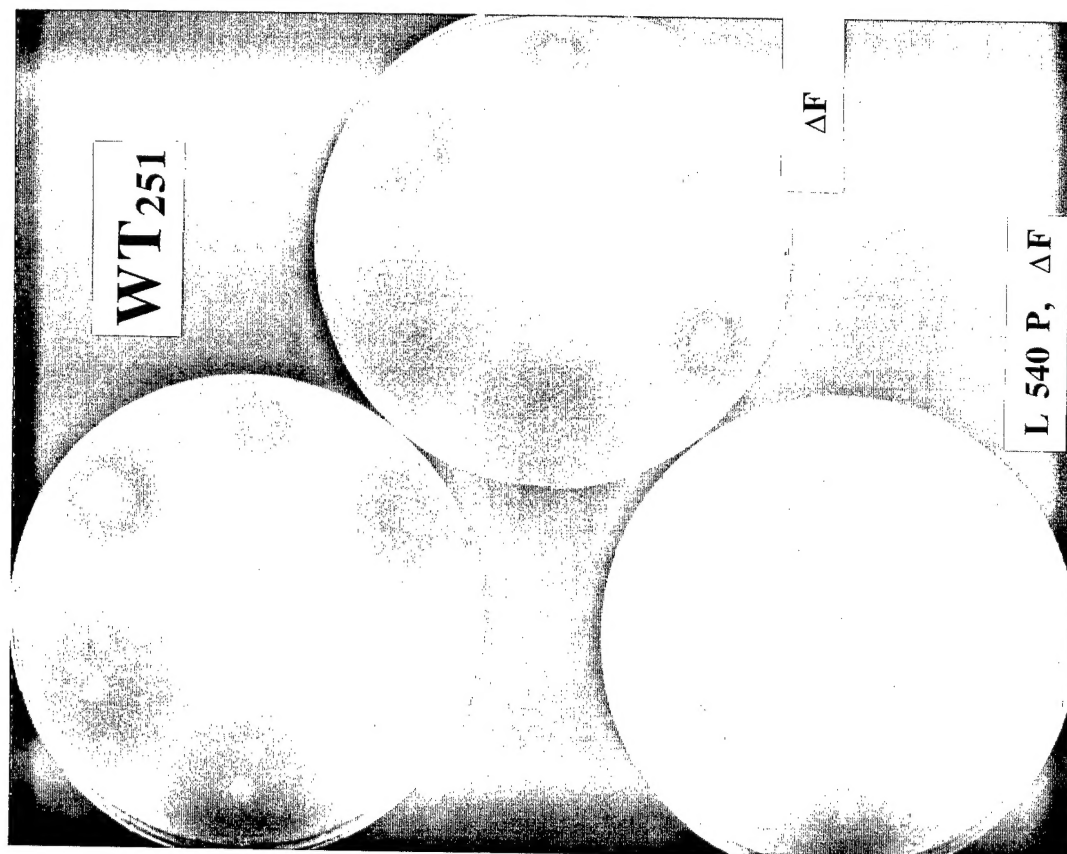
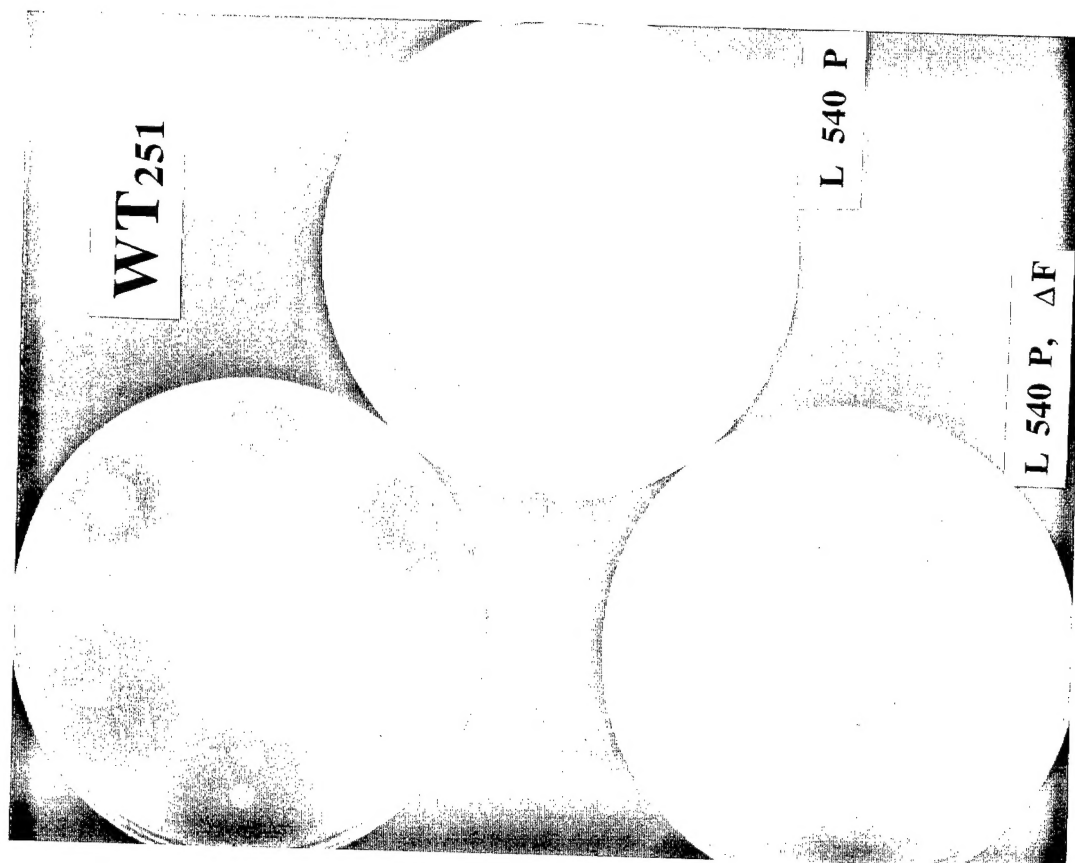


Figure 10.

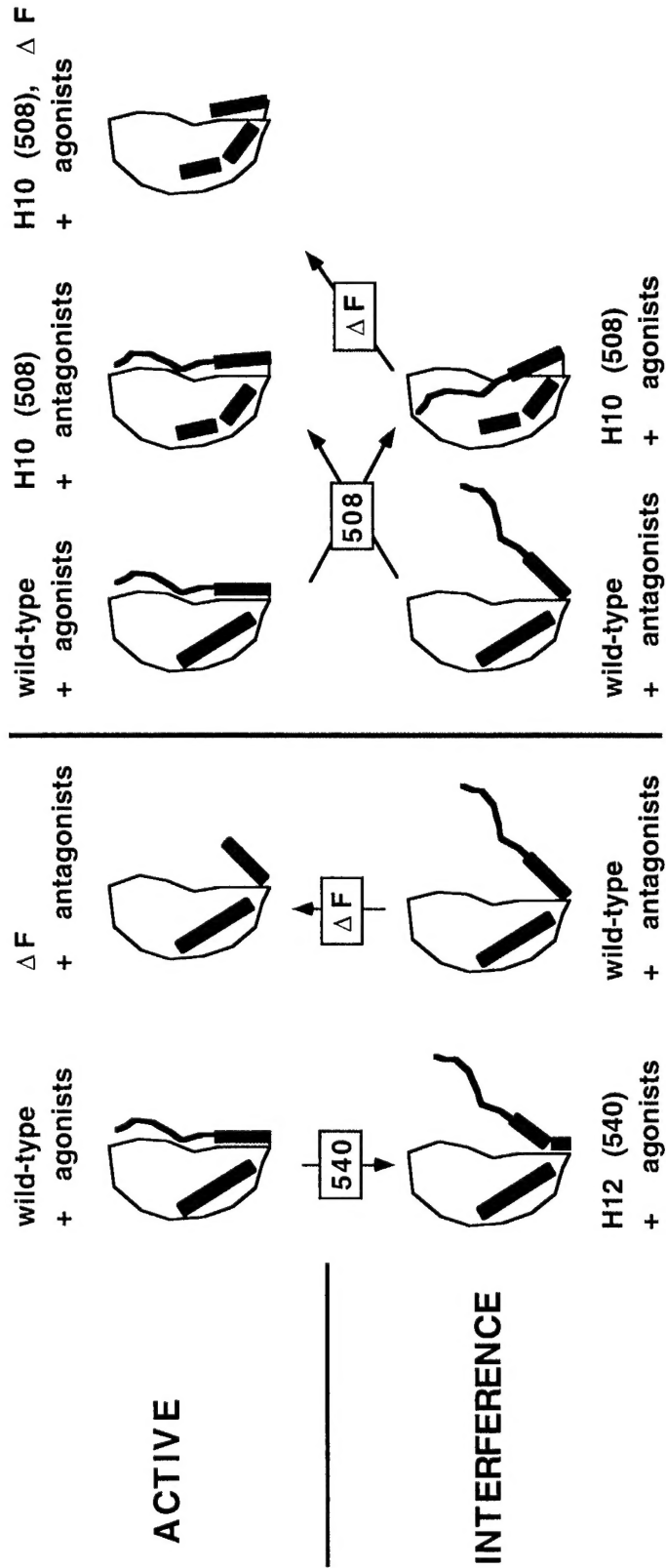


Figure 11.

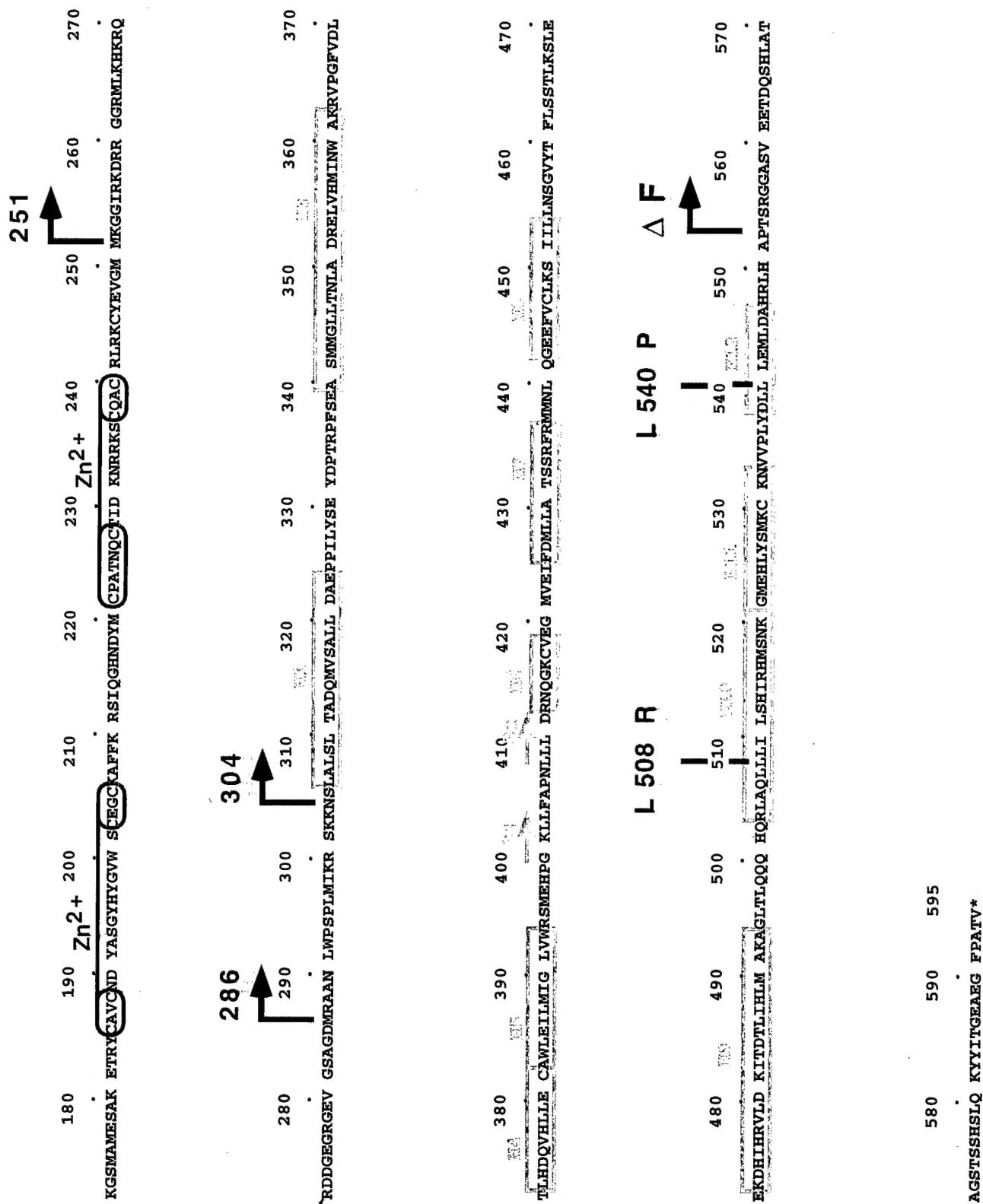




Figure 12.

